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Effects of high glucose on the production of heparan sulfate proteoglycan by mesangial and epithelial cells

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Effects of high glucose on the production of heparan sulfate proteoglycan by mesangial and epithelial cells. Changes in heparan sulfate metabolism may be important in the pathogenesis of diabetic nephropathy. Recent studies performed on renal biopsies from patients with diabetic nephropathy revealed a decrease in heparan sulfate glycosaminoglycan staining in the glomerular basement membrane without changes in staining for heparan sulfate proteoglycan-core protein. To understand this phenomenon at the cellular level, we investigated the effect of high glucose conditions on the synthesis of heparan sulfate proteoglycan by glomerular cells *in vitro*. Human adult mesangial and glomerular visceral epithelial cells were cultured under normal (5 mM) and high glucose (25 mM) conditions. Immunofluorescence performed on cells cultured in 25 mM glucose confirmed and extended the *in vivo* histological observations. Using metabolic labeling we observed an altered proteoglycan production under high glucose conditions, with predominantly a decrease in heparan sulfate compared to dermatan sulfate or chondroitin sulfate proteoglycan. N-sulfation analysis of heparan sulfate proteoglycan produced under high glucose conditions revealed less di- and tetrasaccharides compared to larger oligosaccharides, indicating an altered sulfation pattern. Furthermore, with quantification of glomerular basement membrane heparan sulfate by ELISA, a significant decrease was observed when mesangial and visceral epithelial cells were cultured in high glucose conditions. We conclude that high glucose concentration induces a significant alteration of heparan sulfate production by mesangial cells and visceral epithelial cells. Changes in sulfation and changes in absolute quantities are both observed and may explain the earlier *in vivo* observations. These changes may be of importance for the altered integrity of the glomerular charge-dependent filtration barrier and growth-factor matrix interactions in diabetic nephropathy.

Diabetic nephropathy (DN) affects approximately 30 to 40% of patients with type I diabetes mellitus [1, 2]. Histological hallmarks of this complication include glomerular basement membrane (GBM) thickening, mesangial expansion and sclerosis due to increased production of extracellular matrix (ECM). This increase in ECM is mainly due to an increased amount of collagen type IV, laminin and fibronectin, while heparan sulfate proteoglycan (HSPG) is relatively or absolutely decreased [3]. A decrease in negatively charged molecules within the glomerular basement membrane is thought to be responsible for the albuminuria seen in diabetic nephropathy [4]. HSPGs are believed to be important in this respect, since they consist of sulfate groups distributed along glycosaminoglycan side chains and these may, among other molecules, maintain the negative charge in the GBM [5–7]. HSPG is also an inhibitor of mesangial proliferation, an effect which is dependent on the charge distribution along the glycosaminoglycan (GAG) chains of HSPG [8–10]. Vernier et al [4] showed a reduction of HS associated anionic sites in the lamina rara externa and interna of the GBM in advanced diabetic nephropathy by ultrastructural analysis with cuprolinic blue staining. Removal of HSPG by digestion with heparitinase leads to increased permeability for both ferritin [6] and albumin [5], and injection of antibodies to HS leads to an acute selective proteinuria in rats [11]. Recent immunofluorescence studies by our group on renal biopsies from patients with DN revealed a decrease in HS staining intensity in the GBM without changes in HSPG-core protein staining [3]. Several studies in humans [12, 13] and models of experimental diabetes [14–17] have revealed changes in, or a decrease of, HS production in glomeruli. However, it still remains unclear whether these changes of HS are absolute or relative. Furthermore, it is unknown whether changes concerning the glycosaminoglycan part of the molecule or its sulfation occur. Since the glomeruli in the kidney consist largely of three cell types, that is, endothelial, epithelial, and mesangial cells, cultures of these cells could provide for a useful tool to investigate the proteoglycan production under high glucose conditions *in vitro*. We have recently shown that glomerular visceral epithelial cells (GVEC) and mesangial cells (MC) each have their characteristic pattern of proteoglycan production and differ in heparan sulfate quantity [18]. Especially, GVEC are thought to be important in this respect, since these cells are most likely the major source of proteoglycans incorporated in the lamina externa of the glomerular basement membrane [19].

We hypothesize that high glucose induces changes in HS production, that could differ between different cell types within the glomerulus. Therefore, we cultured GVEC and MC from human adult kidneys and investigated their proteoglycan production under normal and high glucose conditions. These studies were performed using immunofluorescence and metabolic labeling of the cells with both $Na_2{}^{35}SO_4$ and ^{3}H-glucosamine or $Na_2{}^{35}SO_4$ and ^{3}H-leucine, followed by extensive purification steps. Qualitative HS alterations (N-sulfation) were assessed by

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analyzing G-25 gel filtration profiles after HNO_2 (pH 1.5) treatment. Furthermore, an inhibition ELISA using monoclonal antibodies directed against GBM HS was used to measure quantitative differences in HS production.

Methods

Cell culture

MC and GVEC were cultured from glomeruli obtained from normal adult human kidneys that could not be used for transplantation because of anatomical reasons. Methods used to culture MC and GVEC have been published previously [18, 20, 21]. The cells were characterized by their morphology and characteristic immunofluorescence staining. In brief, MC were subcultured from "hillocks," usually appearing after three weeks of outgrowth of the glomeruli, in 24 well plates (Greiner, Frickenhausen, Germany) in DMEM supplemented with 10% heat-inactivated fetal calf serum (Δ FCS; Hyclone Laboratories Inc., Logan, UT, USA), trypsinized, and seeded into T 25 or T 75 flasks. GVEC were passaged immediately after outgrowing from the glomeruli (one week) using PBS-20 mM EDTA into a T 25 or T 75 flask and grown in DMEM with 5% Δ FCS. Characterization of MC was done on the basis of: (a) their cell morphology (multilayer, spindle shaped); (b) uniform fluorescence with FITC-phalloidin for actin and positive staining with monoclonal antibodies against vimentin; (c) absence of staining using monoclonal antibodies TN9, TN10 [22] and anti-von Willebrand factor, anti-cytokeratin and anti-desmin. The monoclonal antibodies TN9 and TN10 specifically recognize proximal tubular epithelial cells and GVEC [22], respectively, and were a gift of Drs. G. Müller and M. Nesper (Medizinische Klinik, Tübingen, Germany). Characterization of GVEC was done on the basis of: (a) their cell morphology (confluent monolayer of polygonal cells); (b) positive staining with monoclonal antibodies TN10, anti-cytokeratin (RGE3) (Eurodiagnostics, Apeldoorn, The Netherlands) and anti-CALLA (Dakopatt, Denmark); (c) absence of staining using monoclonal antibodies TN9, anti-von Willebrand factor and anti-desmin. Experiments were performed in "normal" (5 mM = DMEM + 0.5% Δ FCS) glucose and "high" glucose concentrations (25 mM = DMEM + 0.5% Δ FCS + 20 mM D-glucose). Glucose concentrations were checked using a glucose analyzer 2 (Beckman, USA) before and after each experiment. As an osmotic control we used an L-glucose (DMEM + 0.5% Δ FCS + 20 mM L-glucose) containing medium. No insulin was added to either control or high glucose media. In addition, we measured the insulin concentration (by Radioimmunoassay, Medgenix, Brussels, Belgium; detection limit 3 mU/liter; coefficient of variation at different levels below 6%) in both control and high glucose media containing 0.5 or 2.0% Δ FCS, and did not find detectable levels of insulin.

Immunofluorescence

Cells were grown on sterile glass coverslips (10,000 cells/well) in DMEM containing 10% Δ FCS. After 24 hours the cells were starved in DMEM containing 0.5% Δ FCS for 24 hours. Subsequently, cells were grown for three days in DMEM with 0.5% Δ FCS alone (5 mM glucose), 25 mM glucose, or 25 mM L-glucose. The cells were washed three times with phosphate buffered saline (PBS), fixed in acetone for five minutes at 0°C, air-dried, and immunofluorescent double staining was performed by incubating with anti-HS monoclonal antibodies (JM 403) [11] and anti-HSPG core (B31) polyclonal antibodies [23] for 30 minutes. After extensive washing with PBS, the cells were incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated rabbit anti-goat IgG (RAG IgG-TRITC) (Nordic) followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM (GAM IgM-FITC) for 30 minutes in the dark. GAM IgM-FITC and RAG IgG-TRITC were used as controls. After washing three times with PBS, the slides were mounted in 1,4 diazobicyclo(2, 2, 2)octane-glycerol (DABCO-glycerol) and assessed for fluorescence at 340 to 380 nm (FITC) or 560 nm (TRITC) and photographed through a Leitz microscope (Wetzlar, Germany). The slides were examined and scored without knowledge of the sample origin by two independent observers. Furthermore, intensity of staining was scored on a semiquantitative scale using the exposure time (in seconds) of the camera as measure.

Metabolic labeling

MC and GVEC were cultured in T 75 flasks until confluence and cultured overnight with DMEM supplemented with 2% Δ FCS. Metabolic labeling of cells in media supplemented with a dose response of Δ FCS revealed that a 2% concentration induced maximal incorporation of the label (data not shown). Media were replaced by sulfate free DMEM or sulfate and leucine free DMEM with 2% Δ FCS and labeled for 24 hours at 37°C using 100 μCi/ml $Na_2{}^{35}SO_4$ (carrier-free; New England Nuclear, Boston, MA, USA) and 25 μCi/ml ^{3}H glucosamine or 50 μCi/ml DL-(4, 5)-^{3}H leucine (Amersham International, UK). Cell labeling was performed in different culture conditions: (a) 5 mM glucose; (b) 25 mM glucose; and (c) 25 mM L-glucose (osmotic control). After 24 hours the cell media were separated from the cells. The cells were washed three times with cold PBS and these washes were added to the cell media fraction. Cells were scraped into an extraction buffer using 4 M guanidine HCl at 4°C with the following protease inhibitor cocktail: 10 mM EDTA and 5 mM phenylmethyl sulfonyl fluoride (PMSF), 2 mM benzamidine, 50 mM ε-aminocaproic acid, 0.5 U/ml aprotinin and 5 mM iodoacetamide (all from Sigma, St. Louis, MO, USA). Both the cell media and cell extracts were extensively dialyzed against 0.5 M sodium acetate pH 5.8 with protease inhibitor cocktail at 4°C. The fractions were finally dialyzed against DEAE buffer containing 6 M urea, 50 mM Tris, 0.2% chaps with protease inhibitors cocktail, pH 7.0, and first separated on a 5 ml DEAE anion exchange chromatography column. For the second and final purification the labeled material was separated using HPLC with a DEAE anion exchange 75 × 7.5 mm Bio-Gel TSK DEAE-5PW HPLC column (Biorad, Richmond, CA, USA). The bound material was eluted with an increasing NaCl gradient in DEAE buffer (0.1 to 1.0 M), at a flow rate of 1 ml/min, with 1.0 ml fractions collected. The conductivity of each fraction was measured using a CDM3 radiometer conductivity meter (Copenhagen, Denmark) and the ^{35}S and ^{3}H radioactivity were measured by adding aliquots of the fractions to a fluorophore Ultima Gold (Packard). The separation protocol resulted in different proteoglycan peaks. These peaks were dialyzed against distilled water, freeze dried and stored at −20°C until use. Characterization of the different macromolecules was performed as described previously [18]. Briefly, each of the pooled fractions were treated with 0.75 M NaOH and 20 mM sodium borohydride ($NaBH_4$) (alkaline β elimination) for one hour at 72°C [24] followed by separation on a G50 size separation column

run in 0.5 M sodium acetate buffer containing 0.2% chaps and protease inhibitor cocktail, pH 7.0. The G50 column was presaturated with 1 mg/ml heparin and 10 mg/ml bovine serum albumin (BSA). The void volume was tested for its sensitivity to chondroitinase ABC (Ch-ABC; 2.5 mU), chondroitinase AC (Ch-AC; 2.5 mU) and nitrous acid pH 1.5 (HNO_2) [25–27]. Identification of the $^{35}S/^{3}H$ glycosaminoglycans was determined by the following criteria: (a) molecules with sensitivity to HNO_2 pH 1.5 without sensitivity to Ch-ABC or Ch-AC are designated as heparan sulfate; (b) molecules with sensitivity to Ch-ABC without sensitivity to Ch-AC were designated as dermatan sulfate; and (c) molecules equally sensitive to both Ch-ABC and Ch-AC were referred to as chondroitin sulfate.

Distribution of oligosaccharides formed by nitrous acid cleavage

HSPG isolated from the cell media and cell extracts of GVEC were treated with papain (1 mg/ml) for 18 hours at 55°C, and subsequently heat inactivated at 100°C [28]. The material was centrifuged (3000 rpm) to spin down the papain. The supernatant was cleaved with HNO_2 at pH 1.5, and separated into di-, tetra-, and higher oligosaccharides by gel filtration on a G25 sephadex size separation column (0.95 × 100 cm) in 0.5 M NH_4HCO_3 (2 ml/hr) [29].

Heparan sulfate ELISA

HSPG were detected using an inhibition ELISA with mouse monoclonal antibodies that specifically recognize GBM HS [30]. Cells were cultured in 12 well plates and starved overnight using DMEM with 0.5% Δ FCS. Experiments were performed in 0.5% Δ FCS to induce quiescence of the cells and to minimize the background determination in ELISA. Before and after each experiment cell numbers were determined in triplicate using a Coulter Counter (Coulter Electronics, Mijdrecht, The Netherlands). Cells were cultured for three days in (a) DMEM with 0.5% Δ FCS alone (5 mM glucose), (b) 25 mM glucose or (c) 25 mM L-glucose (osmotic control). HS production of four different cell lines was measured in triplicate and expressed as $\mu g/10^6$ cells/72 hours. Since MC and GVEC produce the complement component factor H, this molecule was used as a control for "normal protein" synthesis using a sandwich ELISA [31]. Cell death was determined by quantification of the lactate dehydrogenase (LDH) levels in the cell media.

Statistical analysis

The data of control and experimental groups were expressed as means ± SD. For statistical analysis we used the Student's *t*-test for unpaired samples, and a P value < 0.05 was considered significant.

Results

Immunofluorescence

Immunofluorescence performed on normal cultured MC resulted in a primarily intracellular staining with an filamentous pattern using both anti-HS and anti-HSPG antibodies. This staining could be inhibited by addition of HS. Intensity of HS staining decreased significantly ($P < 0.002$) (exposure time (in seconds) 1.4 ± 0.01 to 3.3 ± 1.1) after culturing cells in 25 mM glucose, when compared to cells cultured in 5 mM glucose or 25 mM L-glucose (Fig. 1 A, C). In contrast, no difference of staining intensity was observed in 5 mM glucose and 25 mM glucose treated cells using anti-HSPG core antibodies (exposure time 9.6 ± 0.6 to 10.6 ± 1.3; Fig. 1 B, D). GVEC showed predominately intracellular staining for both antibodies. The intensity of HS staining after culturing these cells under high glucose (25 mM glucose) conditions was reduced ($P < 0.02$) (exposure time 45.5 ± 4.2 to 89.6 ± 10.5) when compared to normal (5 mM glucose) conditions (Fig. 2 A, C). Staining of the cells grown in 25 mM L-glucose did not result in a change of intensity. Also, staining of cells with anti-HSPG core antibodies did not show any difference irrespective of the glucose concentration (exposure time 48.6 ± 1.9 to 41.5 ± 3.4; Fig. 2 B, D)

Metabolic labeling

MC and GVEC were labeled as described in **Methods**. The cell media and cell extracts were harvested and the proteoglycans were purified as described. The chemical characterization of the different proteoglycans obtained, has been described previously [18]. Briefly, cell media of MC (Fig. 3 A-C) contained one glycoprotein and the proteoglycans HS (I) (51% of the total ^{35}S-proteoglycan production) and dermatan sulfate (II) (49%). The cell extracts of these cells consisted of (I) HS (58%) and dermatan sulfate (42%) (II) (Fig. 3 D-F). The cell media of GVEC (Fig. 4 A,B) consisted of three glycoproteins and the proteoglycans HS (I) (62%) and a hybrid molecule of both chondroitin sulfate and HS (38%)(II). The cell extracts contained the proteoglycans HS (I) (79%) and chondroitin sulfate (II)(21%) (Fig. 4 C,D). The nature of HSPG produced by GVEC and MC was studied in detail [18] and are important for the interpretation of this study. In brief, by SDS-PAGE we found a 400 kD core protein of HSPG isolated from both the cell media and cell extracts of GVEC. This molecular weight of 400 kD correlated well with the molecular weight of perlecan, the large basement membrane HSPG. Furthermore, expression of this type of HSPG was found using RT-PCR. Peak fractions containing HSPG (as documented by sensitivity to HNO_2 treatment) reacted positive in an ELISA system using antibodies recognizing perlecan. Finally, HSPG isolated from both the cell media and cell extracts of GVEC and MC were subjected to size separation chromatography using a HR 200 sephadex column (HPLC). HSPG isolated from GVEC and MC eluted at a Kav of 0.047 and 0.058, respectively, at which molecules with a molecular weight around 400 kD normally elute.

Absolute differences in proteoglycan production cannot be given with the methods used for isolation since we had to employ two purification steps, first a 5 ml DEAE column and finally a HPLC-DEAE column. The first purification gave variable recoveries. Therefore, we compared production of HS to other proteoglycans. This was done by determining the ratio of proteoglycan production of HS (peak I) over other proteoglycans (peak II). Culturing MC under high glucose conditions resulted in the following changes:

(1) HPLC profiles of the cell media were altered (Fig. 3 A, B), as reflected by decreased $Na_2{}^{35}SO_4$ ratios and ^{3}H glucosamine (peak I/II). Mean ratios of three experiments showed a 2.2-fold decrease for the $Na_2{}^{35}SO_4$ (1.1 ± 0.3 to 0.5 ± 0.1) and 2.3-fold decrease for ^{3}H glucosamine (1.3 ± 0.3 to 0.6 ± 0.1) ratios (Table 1).

(2) The HPLC profiles of the cell extracts changed (Fig. 3 D, E). The mean $Na_2{}^{35}SO_4$ ratio (peak I/II) did not change (1.4 ± 0.1

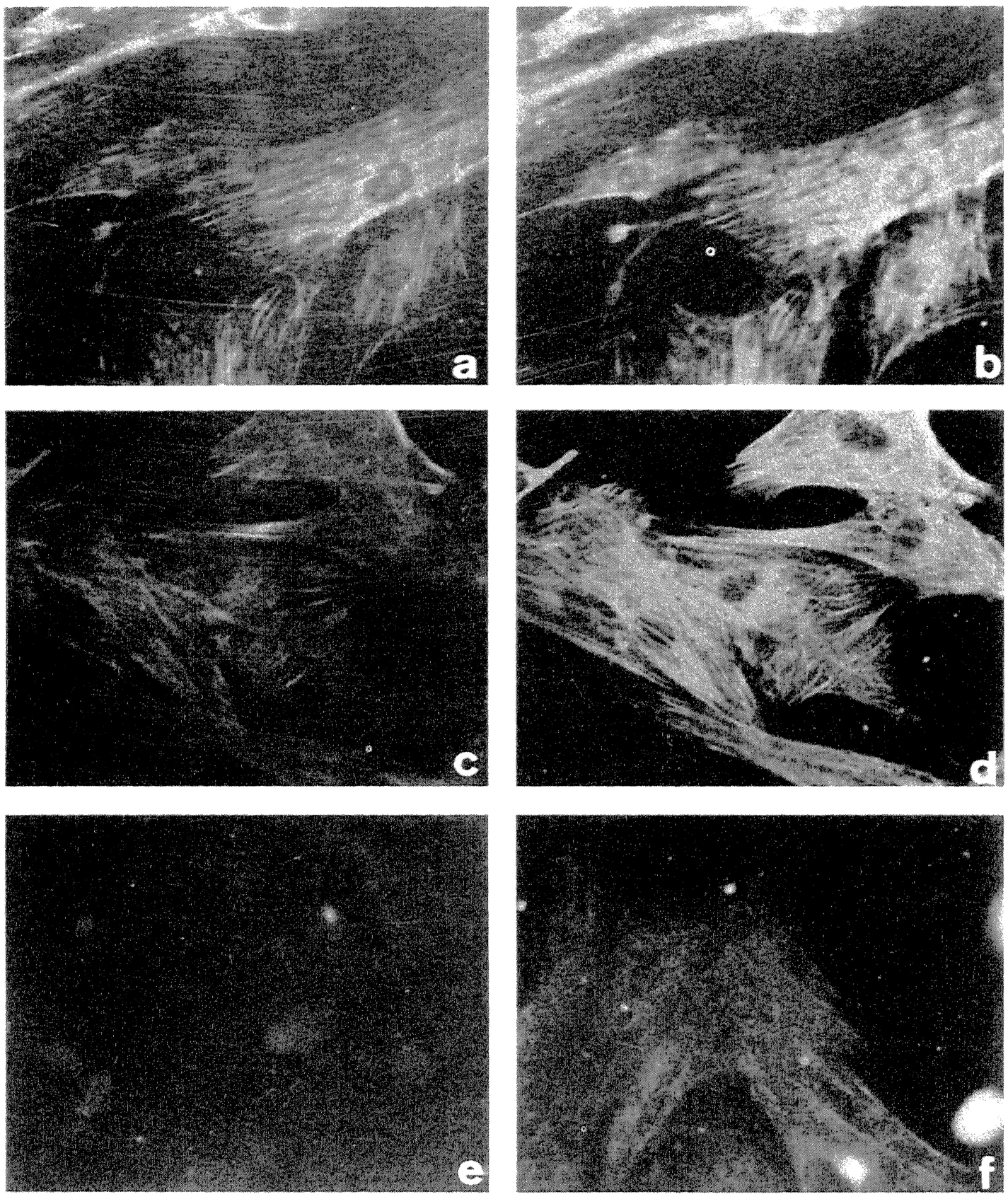


Fig. 1. *Immunofluorence pattern of mesangial cells cultured under normal or high glucose conditions.* Cells were grown on sterile coverslips, fixed with acetone and subsequently stained with either anti-HS monoclonal antibodies (**a**, control; **c**, 25 mM glucose) or with anti-HSPG-core polyclonal antibodies (**b**, control; **d**, 25 mM glucose). GAM IgM-FITC (**e**) and RAG IgG-TRITC (**f**) were used as negative controls.

to 1.3 ± 0.1 ($N = 3$), whereas the ^{3}H glucosamine ratio (peak I/II) decreased 1.8-fold (1.2 ± 0.1 to 0.7 ± 0.2; Table 1).

When GVEC were cultured under high glucose conditions we observed the following changes:

(1) HPLC profiles of the cell media were not altered (Fig. 4 A, B). No significant changes were observed for the mean $Na_2{}^{35}SO_4$ (peak I/II) ratios (1.7 ± 0.3 to 1.5 ± 0.2) and the ^{3}H glucosamine ratios (peak I/II; 1.8 ± 0.5 to 1.8 ± 0.6; Table 2).

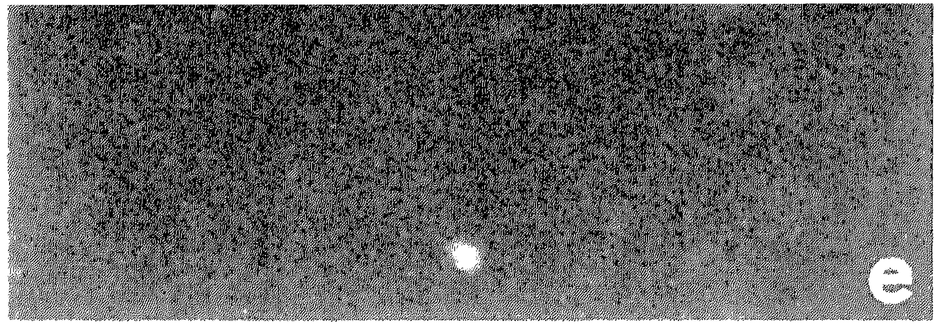
(2) Analysis of the cell extracts of these cells revealed an alteration of the HPLC profile (Fig. 4 C, D). The three different cell lines behaved differently in this respect since the $Na_2{}^{35}SO_4$ ratio changed from 3.7 to 0.7, 3.9 to 2.6 or 1.1 to 0.6, respectively (Table 2). The ^{3}H glucosamine (peak I/II) ratios changed from 3.8 to 0.8, 3.9 to 2.8 and 1.1 to 0.6 in the three cell lines that were tested.

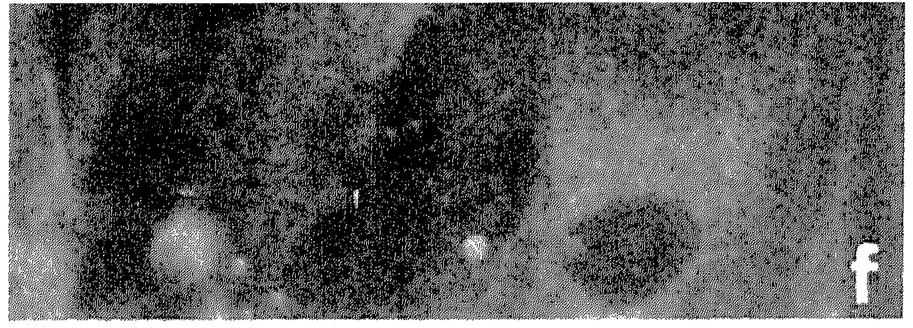
To compare the effects of high glucose on changes in HS to HSPG core protein synthesis, we metabolically labeled the cells with $Na_2{}^{35}SO_4$ and ^{3}H-leucine. Unfortunately, GVEC hardly incorporated ^{3}H-leucine in the proteoglycans they produced (data not shown) as has been published previously [25]. Therefore, the profiles obtained from these experiments are only depicted for MC. Compared to controls, exposure of MC (Fig. 3C) to 25 mM glucose resulted in a 1.4-fold decrease in ratio of ^{3}H-leucine HS/DS (peak I/II) from 1.5 to 1.1. The cell extracts (Fig. 3F) analysis revealed no change in ^{3}H leucine HS/DS (peak I/II) ratio (1.1 to 1.1).

Distribution of oligosaccharides formed by nitrous acid cleavage

HS isolated from the cell media and cell extracts of GVEC grown in 5 or 25 mM glucose media were separated as described in methods. Nitrous acid treatment at pH 1.5 separated HS in di- (peak III), tetra-(peak II) and larger (peak I) oligosaccharides (Fig. 5). Peaks I, II and III were combined as shown in Figure 5 and analyzed for ^{3}H glucosamine (Table 3). When no discernible peak was obtained we calculated the cpm in the fraction which eluted at the same Kav as the original peak fraction (represents molecules with the same molecular weight).

Around 66% of the ^{3}H labeled HS isolated from the cell media of GVEC grown in 5 mM glucose cleaved into di- and tetrasaccharides, whereas 34% was either not cleaved or cleaved into larger fragments. HS obtained from GVEC (cell media) grown with 25 mM glucose resulted in a different cleavage pattern that is, di- and tetrasaccharides (51%) and larger fragments (49%). Nitrous acid treatment of HS obtained from the cell extracts of GVEC (5 mM) resulted in 55% di- and tetrasaccharides and 45% of larger oligosaccharides. HS obtained from the extract of high





stained with either anti HS monoclonal antibodies (**a**, control; **c**, 25 mM glucose) and with anti HSPG-core polyclonal antibodies (**b**, control; **d**, 25 mM glucose). GAM IgM-FITC (**e**) and RAG IgG-TRITC (**f**) were used as negative controls.

glucose treated cells resulted in the same distribution between higher and di- and tetrasaccharides. However, the distribution between di- and tetrasaccharides was altered. The percentage disaccharides changed from 24 to 11% and the tetrasaccharides increased from 31 to 43%.

Heparan sulfate ELISA

MC and GVEC synthesize *de novo* heparan sulfate, since addition of cycloheximide blocks the production [18]. MC produced between 29.8 to 40.7 $\mu g/10^6$ cells/72 hr ($N = 4$), whereas GVEC produced between 216.7 and 282.3 $\mu g/10^6$ cells/72 hr ($N = 4$; Table 4). Both MC and GVEC produced less HS when cultured in high glucose conditions. In the presence of increasing concentrations (5 to 25 mM) of glucose, MC produced significantly lower amounts of heparan sulfate from 15 mM glucose and onwards (Fig. 7A). For GVEC we observed a significant decrease in HS production when cultured in 10 mM glucose and higher (Fig. 7B). High glucose induced a two- to sixfold ($P < 0.01$) reduction in HS production by MC (4 different cell lines; Table 4), whereas for GVEC this decrease was twofold ($P < 0.05$; 3 different cell lines). The decrease in HS production of GVEC was more variable since, in one GVEC line we observed a much more pronounced decrease in HS production (Table 4, Fig. 7B). L-glucose did not have any effect on the HS synthesis (data not shown). No differences in factor H production by MC [112.11 ± 8.9 ng/10^6 cells/72 hr (5 mM) and 126.28 ± 2.97 ng/10^6/72 hr; $N = 3$ (25 mM)] or by visceral epithelial cells (20.10 ± 4.96 ng/10^6 cells/72 hr and 22.14 ± 3.31 ng/10^6 cells/72 hr; $N = 3$) cultured in "normal" and "high glucose" conditions were observed. No detectable levels of LDH were observed in both cell types cultured in the presence of 5 or 25 mM glucose.

We attempted to measure HSPG core protein using an ELISA with anti-HSPG core antibodies (JM 72 and B31) [30]. However, even after improvement, this ELISA was not sensitive enough to measure HSPG in the linear part of the standard curve of the ELISA. Therefore, we cannot provide data about the HSPG core production by GVEC and MC.

Discussion

In this study we analyzed whether high glucose concentrations had an influence on proteoglycan production by cultured glomerular cells. We focused primarily on HSPG production, since previous studies have showed abnormal expression, content, sulfation and/or matrix interaction of this type of proteoglycan in the diabetic GBM [3, 4, 13–15, 32]. HSPG in the GBM seem to be relevant for maintenance of the negative charge barrier, since removal of the molecule by digestion with heparitinase leads to increased permeability for both ferritin [6] and albumin [5]. Also

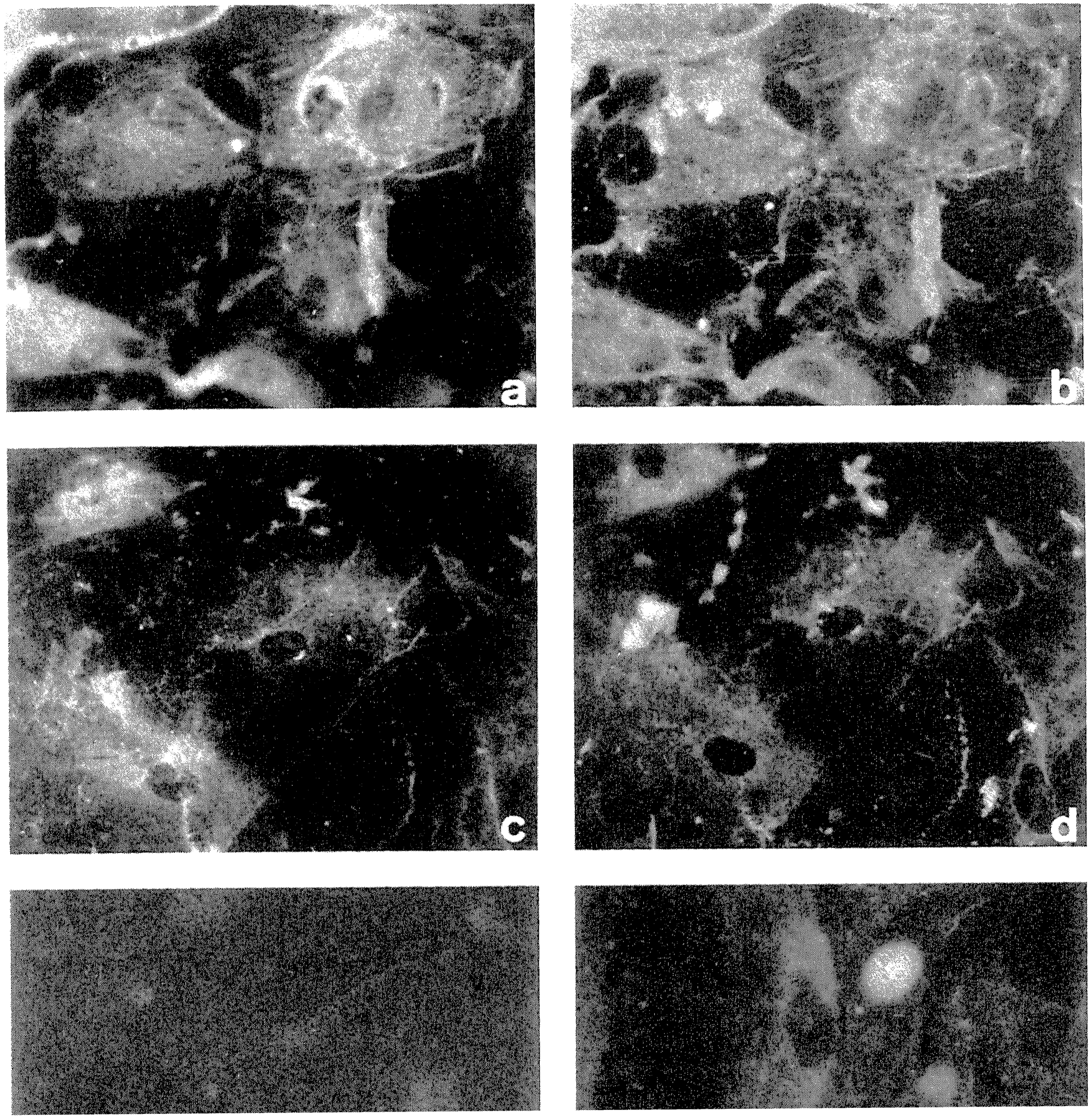


Fig. 2. *Immunofluorence pattern of glomerular visceral epithelial cultured under normal or high glucose conditions.* Cells were grown on sterile coverslips, fixed with acetone and subsequently

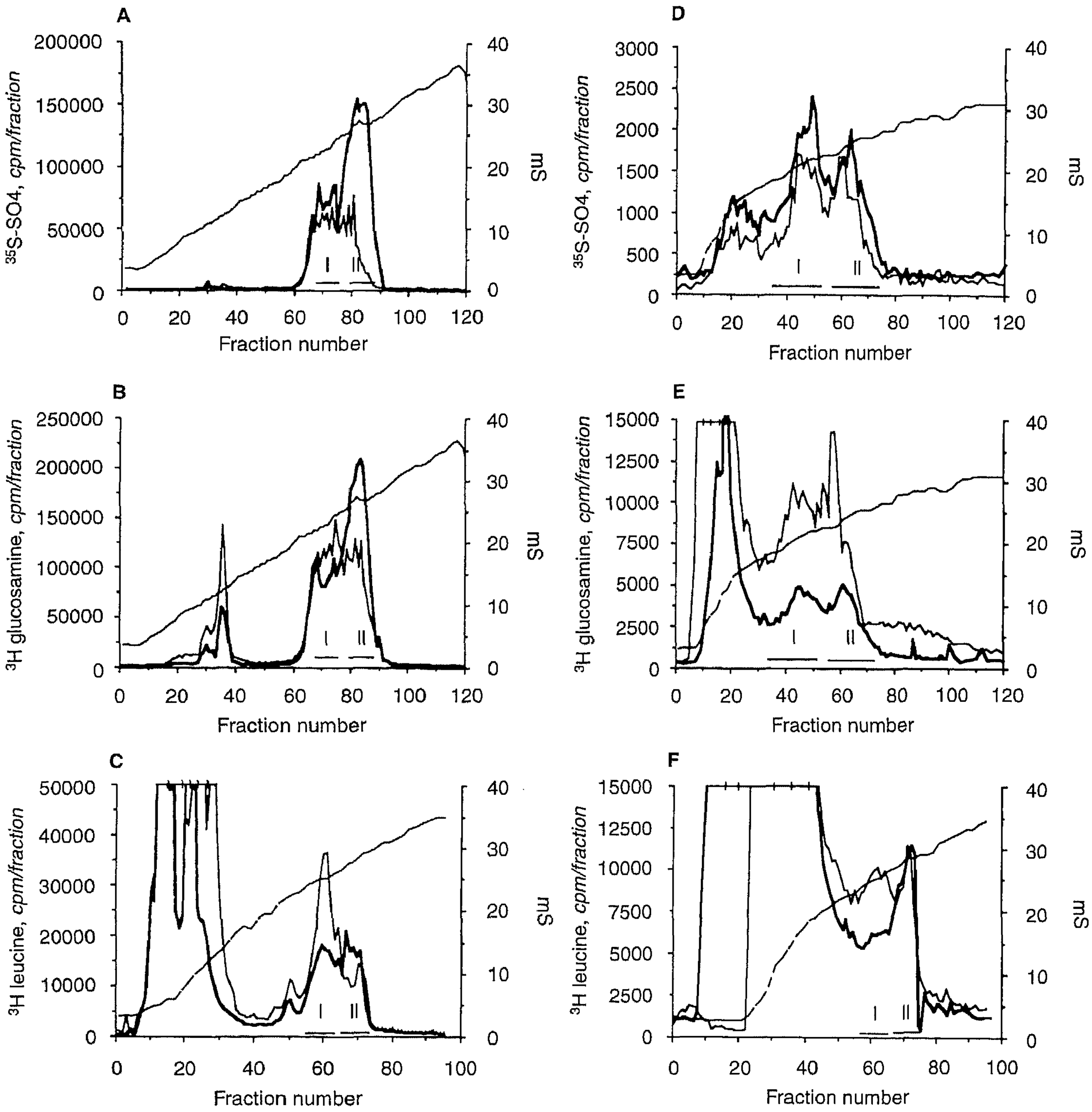


Fig. 3. *HPLC-DEAE chromatography of $Na_2{}^{35}SO_4$ (A, D), ^{3}H-glucosamine (B, E), and ^{3}H-leucine (C, F) labeled macromolecules synthesized by human mesangial cells cultured under normal (——, 5 mM glucose) or high (——, 25 mM glucose) conditions.* Cells were labeled as described and the cell media and cell layers were harvested and extracted. The Figure shows the profile of the macromolecules from the cell media (A, B, C) and cell extracts (D, E, F) that were eluted with a NaCl gradient. The salt gradient is expressed in milliSiemens (· · · · ·, mS).

injection of antibodies to HS leads acutely to a selective proteinuria in rats [11]. Furthermore, HSPG is known to be important in the self assembly and organization of the matrix in the GBM and mesangium. Reduction in the glomerular HSPG content may lead to an increased synthesis of extracellular matrix and thickening of the GBM [32].

Our data support the hypothesis that the diabetic milieu can change renal HS metabolism since high glucose conditions induced an altered HS production by both adult human MC and GVEC *in vitro*. This phenomenon was found using three different methods: immunofluorescence, metabolic labeling and quantification by ELISA. Immunofluorescence clearly showed a decreased staining of HS, for both MC and GVEC when grown under high glucose conditions, whereas staining for the HSPG core protein remained unchanged. These results were consistent with the *in vivo* histological observations in kidneys with diabetic

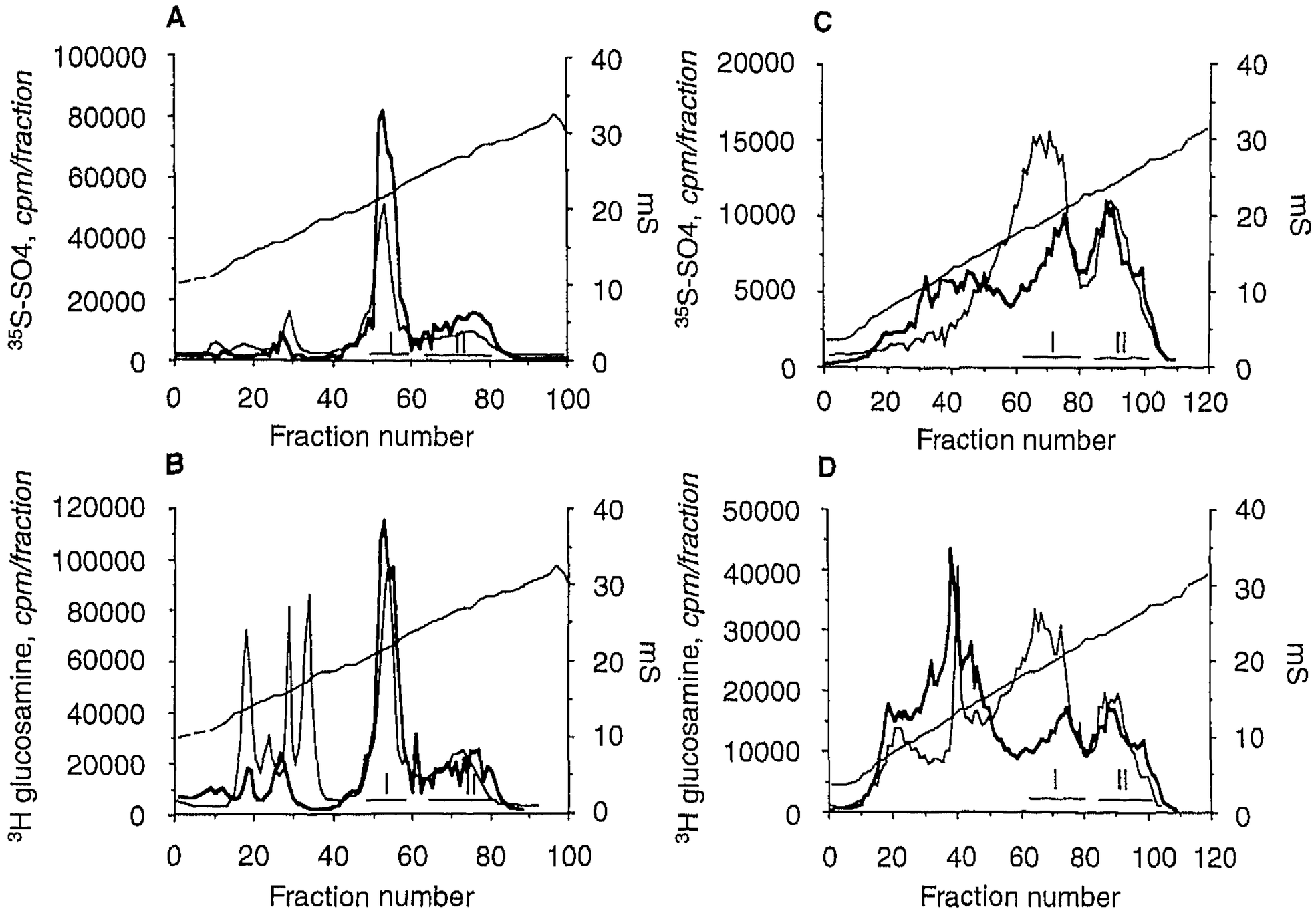


Fig. 4. *HPLC-DEAE chromatography of $Na_2{}^{35}SO_4$ (A, C) and ^{3}H-glucosamine (B, D) labeled macromolecules synthesized by human glomerular visceral epithelial cells cultured under normal (——, 5 mM glucose) or high glucose (——, 25 mM) conditions.* The profiles of the incorporated ^{35}S/^{3}H macromolecules from the cell media (A,B) and cell extracts (C,D) that were eluted with a NaCl gradient are shown. The salt gradient was expressed in milliSiemens (· · · ·, mS).

Table 1. Influence of high glucose (25 mM) on the production of heparan sulfate (HS) and dermatan sulfate (DS) by human mesangial cells both excreted (media) and cell associated (extract)

		HS/DS $Na_2{}^{35}SO_4$		HS/DS ^{3}H glucosamine	
		5 mM[a]	25 mM[a]	5 mM	25 mM
Media	1	0.9	0.5 (1.1)[b]	1.2	0.6 (1.0)[b]
	2	1.0	0.6	1.1	0.7
	3	1.4	0.5	1.6	0.5
Extract	1	1.5	1.1 (1.1)[b]	1.0	0.6 (1.1)[b]
	2	1.3	1.1	1.0	0.9
	3	1.5	1.3	1.7	0.5

Results are expressed as HS/DS ratios as assessed by $Na_2{}^{35}SO_4$ and ^{3}H-glucosamine labeling.
[a] Concentration of glucose in culture medium
[b] Ratios obtained after culture in L-glucose containing media

Table 2. Influence of high glucose (25 mM) on the production of heparan sulfate (HS) and chondroitin sulfate (CS) by human glomerular epithelial cells both excreted (media) and cell associated (extract)

		HS/HSCS $Na_2{}^{35}SO_4$		HS/HSCS ^{3}H glucosamine	
		5 mM[a]	25 mM[a]	5 mM	25 mM
Media	1	2.0	1.6	1.9	1.8
	2	1.6	1.5	2.3	2.4
	3	1.3	1.2 (1.3)[b]	1.3	1.2 (1.1)[b]
		HS/CS $Na_2{}^{35}SO_4$		HS/CS ^{3}H glucosamine	
Extract	1	3.7	0.7	3.8	0.8
	2	3.9	2.6	3.9	2.8
	3	1.1	0.6 (1.1)[b]	1.1	0.6 (1.3)[b]

Results are expressed as HS/CS ratios as assessed by $Na_2{}^{35}SO_4$ and ^{3}H-glucosamine labeling.
[a] Concentration of glucose in culture medium
[b] Ratios obtained after culture in L-glucose containing media

nephropathy [3, 32]. Using the same antibodies, a decreased GBM HS staining was observed in biopsies from patients with overt diabetic nephropathy, while the staining for HSPG core remained unchanged. No differences in staining for HS or HSPG core were observed when cells were cultured with 25 mM L-glucose instead of 25 mM D-glucose. L-glucose is not taken up by cells, but does create the same high osmotic environment. The (unchanged) staining intensity was similar to that of cells grown in 5 mM D-glucose, and indicates that the observed changes were not due to an osmotic effect.

To further investigate the effects of high glucose on the HSPG production compared to HS-GAG, we performed metabolic dual labeling with $Na_2{}^{35}SO_4$ and ^{3}H-glucosamine, to investigate the effect of "high glucose" conditions on $Na_2{}^{35}SO_4$ compared to ^{3}H-labeled glycosaminoglycan side chains, or with ^{35}S-sulfate and ^{3}H-leucine to compare ^{35}S-labeled glycosaminoglycan side chains

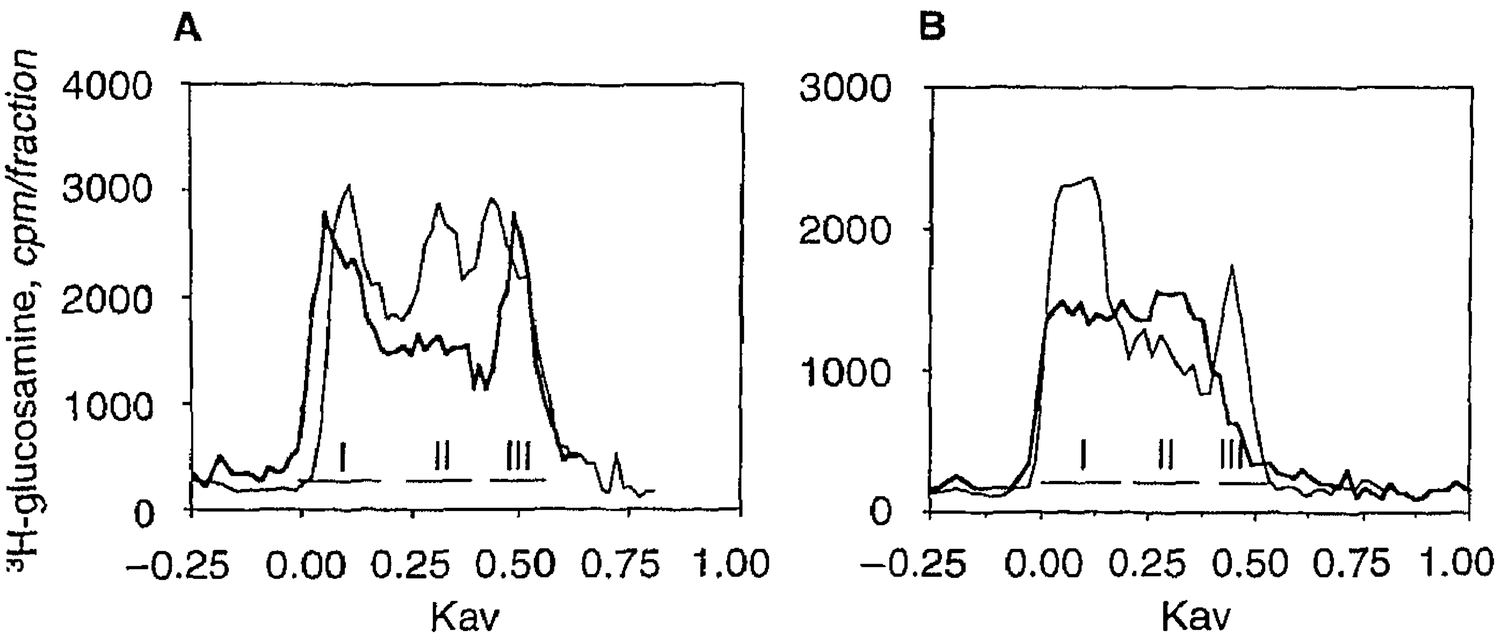


Fig. 5. *G25 sephadex fractionation of heparan sulfate deamination products obtained from glomerular visceral epithelial cells.* Heparan sulfate excreted in the cell media (**A**) and isolated from the cell extracts (**B**) of GVEC grown in 5 or 25 mM glucose were treated with nitrous acid pH 1.5. The products were chromatographed on a 0.95 × 100 cm G25 sephadex column in 0.5 M NH_4HCO_3. Fractions of 1 ml were collected and analyzed for ^3H. Fractions were pooled as indicated by the bars. Fraction III represents the disaccharides, II the tetrasaccharides and I the larger oligosaccharides.

Table 3. Distribution of oligosaccharides formed by nitrous acid cleavage

Size class	Peak[a]	^3H GAG *%total*			
		Cell media		Cell extract	
		5	25	5	25
Di-	III	35	27	24	11
Tetra-	II	31	24	31	43
Higher	I	34	49	45	47

Heparan sulfate isolated from the cell media and cell extract of GVEC were cleaved by nitrous acid pH 1.5. The obtained fragments were separated by G25 gel filtration chromatography.

[a] Figure 5

Table 4. Production of heparan sulfate in four different cell lines of mesangial cells (MC) and visceral epithelial cells (GVEC), cultured under normal (5 mM) or high glucose (25 mM) conditions

Cell line #	Heparan sulfate $\mu g/10^6$ cells/72 hr			
	MC		GVEC	
	5 mM[f]	25 mM[f]	5 mM	25 mM
1	38.9 ± 2.7	18.7 ± 2.8[a]	282.3 ± 88.3	124.3 ± 28.4[c]
2	40.7 ± 5.4	11.3 ± 3.0[b]	276.4 ± 43.9	123.0 ± 70.8[d]
3	29.8 ± 8.0	5.1 ± 2.1[a]	225.6 ± 10.9	115.4 ± 9.2[b]
4	35.2 ± 8.8	8.1 ± 2.8[a]	216.7 ± 38.0	[e]

Heparan sulfate was measured in the cell media using the heparan sulfate inhibition ELISA. HS concentration was measured in triplicate and expressed in $\mu g/10^6$ cells/72 hr.

[a] $P < 0.01$, [b] $P < 0.002$, [c] $P < 0.02$, [d] $P < 0.05$, [e] undetectable, [f] glucose concentration in medium

to the ^3H-labeled core protein. After separation of the proteoglycans by DEAE-anion exchange chromatography, we calculated the ratio of both $Na_2{}^{35}SO_4$ and ^3H incorporation in HS/CSHS or HS/CS for GVEC and HS/DS for MC. With this method we observed no changes in HS secreted in the cell media by GVEC, as compared to the hybrid molecule (CSHS) for the $Na_2{}^{35}SO_4$ or ^3H glucosamine ratios. For MC we observed a decrease in both HS/DS ^3H glucosamine ratio and HS/DS $Na_2{}^{35}SO_4$ ratio and a slight decrease in the ^3H leucine ratio. For both cell types we found decreased HS/DS and HS/CS ^3H glucosamine ratios, respectively, when analyzing the cell extracts. This overall decrease in ratio of HS compared to either DS or CS may indicate either: (a) an absolute decrease in HS production without affecting the DS or CS production; or (b) a decrease in HS and an increase in DS or CS production; or (c) unchanged HS production and increased DS or CS production. The decrease in ^3H leucine HS/DS ratio observed when analyzing the media of MC is difficult to interpret since the HSPG-core protein staining by IF remained unaltered. However, this apparent discrepancy can be explained by the fact that polyclonal antibodies against HSPG core protein predominantly stain the cells intracellularly. This observation is therefore in agreement with the unchanged ^3H leucine HS/DS ratio in the cell extract, which contains both intracellular and extracellular components. The unchanged $Na_2{}^{35}SO_4$ and decreased ^3H glucosamine ratios observed when analyzing the cell extracts of MC could indicate either an oversulfation of HS or undersulfation of DS.

To quantitate the change in HS production by GVEC and MC under high glucose conditions *in vitro*, we measured the HS content in the cell media using a specific GBM HS inhibition ELISA [30]. *De novo* synthesized HS by MC was significantly decreased when cells were cultured in high glucose containing media as compared to control media. This decrease was observed in four different cell lines. For GVEC we observed a less pronounced, but also significant reduction in HS synthesis, when cells were grown in the presence of 25 mM glucose as compared to control media (5 mM glucose). The observed effect was not due to changes in cell numbers, since these did not differ significantly between "normal" and "high" glucose conditions, and furthermore, all HS levels were corrected for cell number. There were no differences in overall protein production as was shown using "factor H" ELISA and viability of the cells (LDH release). A dose response of glucose concentrations revealed a significant decrease in HS production when MC or GVEC were cultured in 15 mM or 10 mM glucose, respectively. Metabolic labeling of GVEC demonstrated that incorporation and excretion of proteoglycans can differ between cell lines which became apparent when we analyzed the cell extracts of these cells. The problem of metabolic labeling lies in the isolation procedures necessary to obtain the different proteoglycans. This makes estimates of quantities in absolute terms rather unprecise. We therefore focused in the metabolic studies on relative changes comparing HS with other glycosaminoglycans. Changes in terms of absolute quantities were only studied by HS ELISA, whereas metabolic labeling not only served as a useful tool to identify the different proteoglycans independently from immunological characterization studies, but also allowed us to study (1) changes in HS production compared with other proteoglycans produced and (2) ratios of sulfate and

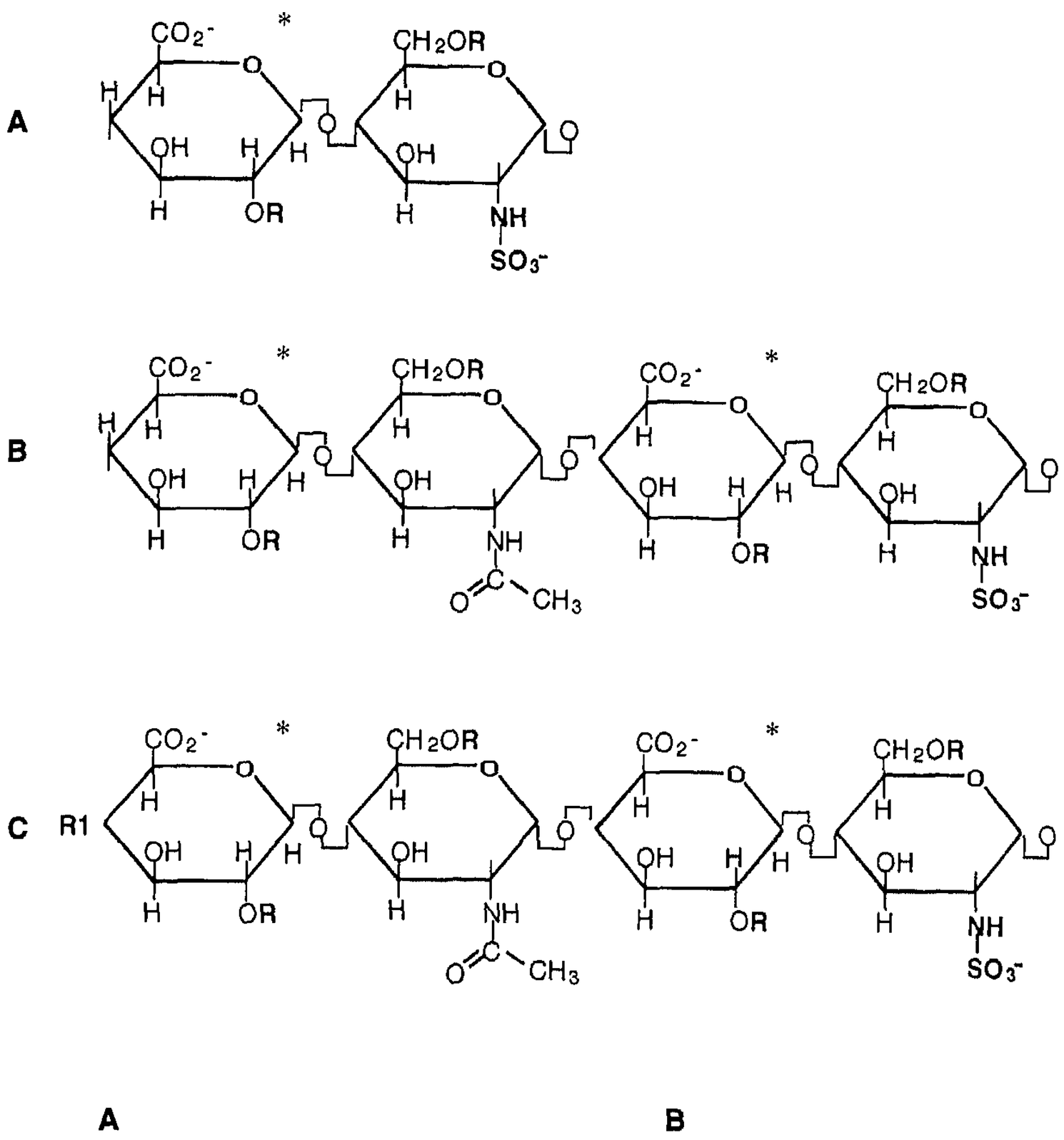


Fig. 6. *The possible heterogeneity of sulfation along the HS-GAG side chains.* Molecules depicted in **A, B** and **C** may all function as building blocks of the side chain. N-sulfation as outlined in A, B and C would result in di-, tetra- or larger saccharides as shown in Figure 5 as fractions III, II, and I, respectively. R = SO_3^-/H; R1 = 6 or more saccharide residues; * either iduronic or glucuronic acid.

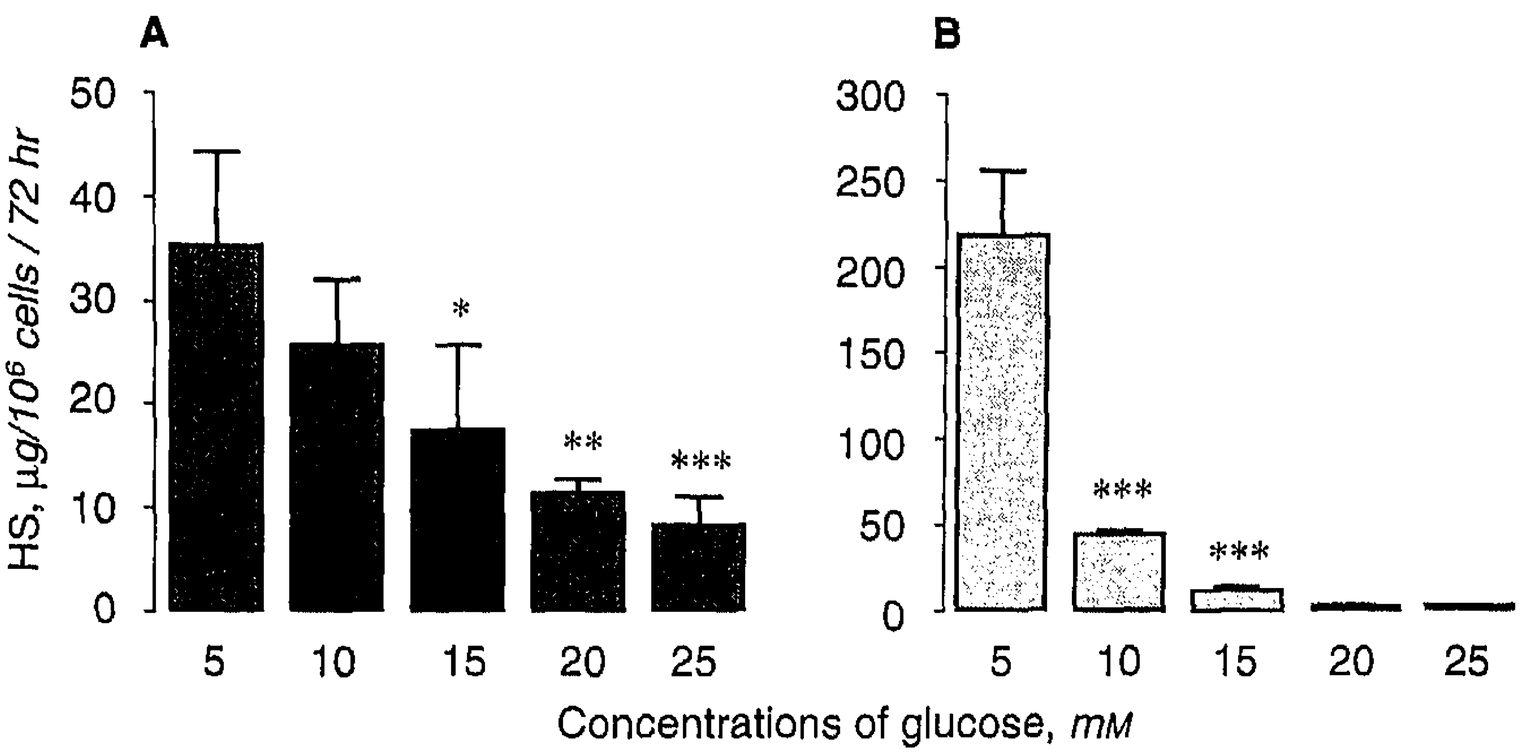


Fig. 7. *The effect of increasing concentrations of glucose on the production of heparan sulfate by* (**A**) *mesangial cells and* (**B**) *glomerular visceral epithelial cells.* MC and GVEC were cultured with a dose response of glucose for three days, cell media was harvested and assessed in the heparan sulfate ELISA in triplicate.

glucosamine incorporation [18]. This last parameter is of interest, since it has been shown that sulfation patterns of GAG side chains may be of significance for the specific interactions of proteoglycans with growth factors [33–35].

Therefore we also studied the heterogeneity in N-sulfation of the oligosaccharides in HS. HS molecules obtained from the cell media and cell extracts of GVEC grown in 5 or 25 mM glucose were cleaved with nitrous acid pH 1.5. Nitrous acid at pH 1.5 specifically cleaves HS in $GlcNSO_3$ residues. To illustrate the underlying mechanisms, relevant chemical structures are given in Figure 6. Disacccharides are obtained from regions of the polymer chain with contiguous N-sulfated disaccharides units, and tetrasaccharides are obtained from regions containing HNO_2-resistant GlcNAc residues [36]. Peaks I, II and III were combined as shown in Figure 5 and analyzed for ^3H glucosamine (Table 3). The size distribution of oligosaccharides of HS obtained from GVEC grown in 25 mM glucose was different from those obtained from GVEC grown in 5 mM glucose. Therefore, we conclude that there is an altered N-sulfation pattern of heparan sulfate produced by GVEC under high glucose conditions.

The biosynthesis of HS is dependent on a variety of processes. The initiating and rate-limiting steps of these processes are the N-deacetylation and subsequently N-sulfation of N-acetylglucosamine; thus, C5 epimerization of D-glucuronic acid to iduronic acid and the many O-sulfation reactions cannot occur until N-sulfation has been initiated [37]. The differences in N-sulfation are compatible with the idea that high glucose induces a change in N-deacetylase activity, a hypothesis which has also been suggested

by others [38, 39]. Unger et al [39] observed that hepatocytes from streptozotocin-diabetic rats expressed less N-deacetylase activity than control rats. Recent studies from Ishihara et al [36] demonstrated that heparan sulfate-N-deacetylase/N-sulfotransferase (NSNdAc/NST) catalyzes both the N-deacetylation and N-sulfation of glucosamine residues in HS. The level of HS-NdAc/NST expression is important in regulating the formation of large fully N-sulfated, highly O-sulfated domains in HS. These domains have high affinity for basic fibroblast growth factor (bFGF) and changes in these domains may therefore be important for cell growth and matrix production. Our results are compatible with the hypothesis that the HS produced under high glucose conditions might interact differently with growth factors because of an altered sulfation pattern.

So far, *in vitro* studies using metabolic labeling, revealed only changes of total labeled proteoglycan production by human fetal MC [40] or rat MC [41]. Studies utilizing metabolic labeling of streptozotocin-induced diabetic rats [14] with $Na_2{}^{35}SO_4$ revealed 30 to 40% decreased incorporation into the glomerular extracellular matrices when compared to control animals. Silbiger et al [41] investigated the total production of proteoglycans by rat MC under normal and high glucose conditions and found no differences. However, these findings could be due to a relative decrease in HS production and a relative increase in DS production, since the production of the individual components was not studied. To our knowledge, no data are available concerning the negative charge of human GBM-HSPG from patients with diabetic nephropathy or diabetes. *In vivo* evidence for an altered GAG synthesis and normal rates of HSPG core protein were described by our group [3, 32]. Data from experimental animals were published by several authors. Klein, Brown and Oegema [42] studied GBM-HSPG isolated from metabolically labeled glomeruli obtained from streptozotocin induced diabetic rats. This group found less ^{35}S-proteoglycans in diabetics compared to control. A decrease of HSPG was only found in the rapidly metabolized pool of HS. However, no changes were found in the hydrodynamic size or charge of these molecules. Evidence for decreased synthesis of basement membrane-specific proteoglycans in diabetes also came from work of Rohrbach et al [15] showing that less HSPG was synthesized by BM-producing tumors (EHS) grown in diabetic mice despite an unchanged protein synthesis. No data concerning the negative charge was given. Finally, Cohen, Klepser and Wu [43] found a decrease in sulfate to uronate ratio in HSPG isolated from the GBM of streptozotocin induced diabetic rats, indicating reduction in charge.

In conclusion, our results demonstrate that high glucose conditions lead to alterations in HS production by human adult GVEC and MC in that there is a decreased production and a changed pattern of N-sulfation. These alterations may cause a decreased concentration of an altered HSPG in the glomerulus and thus lead to albuminuria (by a decreased charge barrier) and mesangial expansion (by a decreased interaction with growth factors such as FGF) observed *in vivo* in diabetic nephropathy.

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Abbreviations

HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; GAG, glycosaminoglycan; DN, diabetic nephropathy; MC, mesangial cells; GVEC, glomerular visceral epithelial cells; DMEM, Dulbecco's modified Eagle's medium.

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